441407UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF CHEMICAL SAFETY
AND POLLUTION PREVENTION

MEMORANDUM

DATE: May 2, 2019

SUBJECT: **Valifenalate**: Report of the Cancer Assessment Review Committee

PC Code: 128200
Decision No.: 530024
Petition No.: N/A
Risk Assessment Type: Cancer Assessment
TXR No.: 0057785
MRID No.: N/A

DP Barcode: D441407
Registration No.: 279-GAGR
Regulatory Action: N/A
Case No.: N/A
CAS No.: 283159-90-0
40 CFR: N/A

FROM: Melinda Wilson, Executive Secretary
Cancer Assessment Review Committee
Health Effects Division (7509P)

THROUGH: Gregory Akerman, Chair
Cancer Assessment Review Committee
Health Effects Division (7509P)

TO: Connor Williams, Toxicologist
RAB IV, Health Effects Division (7509P)

The Cancer Assessment Review Committee met on August 29, 2018 to evaluate the cancer classification of valifenalate in accordance with the *EPA's Final Guidelines for Carcinogen Risk Assessment* (March, 2005). Attached please find the final Cancer Assessment Document.

EVALUATION OF THE CARCINOGENIC POTENTIAL OF
VALIFENALATE

PC Code 128200

August 29, 2018

CANCER **A**SSESSMENT **R**EVIEW **C**OMMITTEE
HEALTH EFFECTS DIVISION
OFFICE OF PESTICIDE PROGRAMS

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EXECUTIVE SUMMARY

On August 29, 2018, the Cancer Assessment Review Committee (CARC) of the Health Effects Division of the Office of Pesticide Programs met to evaluate the carcinogenic potential of the agricultural fungicide valifenalate. Valifenalate is currently undergoing primary registration in the United States and has not previously been classified as to specific carcinogenicity by EPA. The registrant has submitted information in support of a proposed mitogenic mode of action (MOA) for valifenalate-induced liver tumors involving the co-activation of the nuclear receptors peroxisome proliferator activated receptor-α (PPARα), constitutive androstane receptor (CAR) and pregnane X receptor (PXR). The evidence included the following:

1. A carcinogenicity study demonstrating tumorigenesis in mice. (MRID 49807232)

2. A white paper submitted by the registrant proposing PPARα activation as the mode of action involved in liver tumor formation and which illustrates proposed steps involved in cellular injury and eventual carcinogenesis. (MRID 49807234)

3. A series of mechanistic studies submitted by the registrant in support of the proposed mode of action. (MRIDs 49807228, 49807252, 49807218, 49807233)

The CARC concluded that there is sufficient evidence with dose and time concordance to support the postulated MOA for hepatocellular tumors in mice. The CARC considered the following in its weight-of-evidence determination of the carcinogenic potential of valifenalate:

1. The lung tumors seen in female mice were not considered treatment-related;

2. The hepatocellular tumors seen in male and female mice were considered treatment-related;

3. The proposed MOA involving the co-activation of PPARα, CAR, and PXR leading to subsequent cellular proliferation and hepatocellular tumor formation in mice was adequately supported by studies that clearly identified the sequence of key events, dose-response concordance and temporal relationship for this tumor type;

4. There is no mutagenicity concern based on the results from the *in vitro* and *in vivo* genetic toxicity studies; and

In accordance with the EPA's *Final Guidance for Carcinogen Risk Assessment* (March 2005), the CARC classified valifenalate as "Not likely to be carcinogenic to humans at dose levels that do not cause a proliferative response in the liver." Therefore, the Agency has determined that quantification of risk using a non-linear approach (i.e., reference dose (RfD) will adequately account for all chronic toxicity, including carcinogenicity, that could result from exposure to valifenalate.

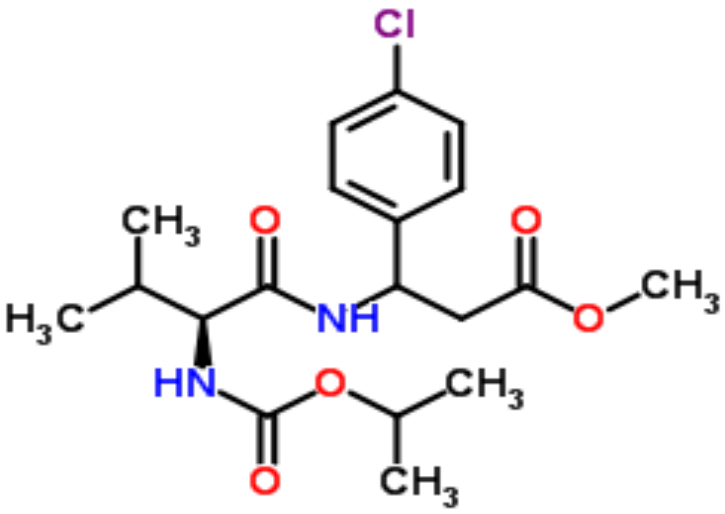
I. INTRODUCTION

On August 29, 2018 the Cancer Assessment Review Committee (CARC) of the Health Effects Division of the Office of Pesticide Programs met to evaluate the carcinogenic potential of valifenalate.

II. BACKGROUND INFORMATION

Valifenalate is a carboxylic acid amide fungicide used on agricultural crops. The active ingredient, methyl N-(isopropoxycarbonyl)-L-valyl-(3RS)-3-(4-chlorophenyl)-β-alaninate affects all growth stages of certain fungi by acting on the enzyme, cellulose synthase, resulting in the inhibition of spore germination, prevention of the development of the germination tube and interference with the biosynthesis of cell walls. Valifenalate is effective in the control of oomycete fungi, which are responsible for common plant diseases such as late blight and downy mildew.

Valifenalate is currently registered globally in the European Union, Mexico, and Latin America on grapes and vegetable crops. Proposed US tolerances include bulb vegetables, cucurbits, fruiting vegetables, celery, and potatoes. The registrant is also pursuing an import tolerance on grapes to allow trade between other countries with active registrations for valifenalate use on grapes.



III. EVALUATION OF CARCINOGENICITY STUDIES

1. *Carcinogenicity Study in Mice*

Citation: Webley L. (2004) IR5885 Carcinogenicity study by dietary administration to CD-1 mice for 78 weeks. Huntingdon Life Sciences Ltd. Woolley Road, Alconbury, PE28 4HS, England, unpublished report. MRID 49807232.

In a 78-week oral carcinogenicity study, valifenalate was administered to Crl: CD-1(ICR)BR mice (50 mice/sex/dose) at concentrations of 0, 150, 850 and 5000 ppm (equivalent to 0, 16.8, 97.2 and 657 mg/kg/day for males and 0, 21.6, 124 and 756 mg/kg/day for females).

Survival Analyses

There were no statistically significant survival disparities among the dose groups in male or female mice (Tables 1 and 2) (L. Brunsman, TXR 0057762, 07/25/2018)

TABLE 1. Valifenalate – Crl:CD-1(ICR)BR Mouse Study
(MRID No. 49807232)

Male Mortality Rates[+] and Cox or Generalized K/W Test Results

	Weeks			
Dose (ppm)	1-26	27-52	53-78 [f]	Total
0	0/50	2/50	15/48	17/50 (34)
150	1/50	8/49	11/41	20/50 (40)
850	0/50	4/50	9/46	13/50 (26)
5000	2/50	5/48	11/43	18/50 (36)

+ Number of animals that died during the interval/Number of animals alive at the beginning of the interval.
[f] Final sacrifice at week 78.

Note: Time intervals were selected for display purposes only.
Significance of trend denoted at control.
Significance of pair-wise comparison with control denoted at dose level.
If *, then $p < 0.05$. If **, then $p < 0.01$.

TABLE 2. Valifenalate – Crl:CD-1(ICR)BR Mouse Study
(MRID No. 49807232)

Female Mortality Rates[+] and Cox or Generalized K/W Test Results

Dose (ppm)	Weeks 1-26	27-52	53-78 [f]	Total
0	0/50	2/50	10/48	12/50 (24)
150	1/50	1/49	8/48	10/50 (20)
850	1/50	4/49	9/45	14/50 (28)
5000	1/50	1/49	13/48	15/50 (30)

+ Number of animals that died during the interval/Number of animals alive at the beginning of the interval.
[f] Final sacrifice at weeks 78-79.
Note: Time intervals were selected for display purposes only.
Significance of trend denoted at control.
Significance of pair-wise comparison with control denoted at dose level.
If *, then $p < 0.05$. If **, then $p < 0.01$.

Tumor Analyses

Male mice had statistically significant trends at $p < 0.01$, and statistically significant pair-wise comparisons of the 5000 ppm (657 mg/kg/day) dose group with the controls at $p < 0.05$, for liver adenomas, carcinomas and combined adenomas and carcinomas. The statistical analyses of the tumors in the male mouse study were based upon Fisher's Exact Test and the Exact Test for Trend (Table 3a). The incidences of adenomas, carcinomas and combined in males all exceed the historical control ranges given by the performing laboratory (Table 3b).

Female mice had statistically significant trends at $p < 0.01$, and statistically significant pair-wise comparisons of the 5000 ppm (756 mg/kg/day) dose group with the controls at $p < 0.05$, for liver adenomas and combined adenomas and carcinomas. There were also statistically significant trends for lung bronchioalveolar adenomas and combined lung bronchioalveolar adenomas and carcinomas, both at $p < 0.05$. There were no statistically significant pair-wise comparisons of the dosed groups with the controls for lung tumors. The statistical analyses of the tumors in the female mouse study were based upon Fisher's Exact Test and the Exact Test for Trend (Tables 4a and 5a) (L. Brunsman, TXR 0057762, 07/25/2018). The incidences for liver adenomas and combined liver adenomas and carcinomas in females exceed the historical control ranges given by the performing laboratory (Table 4b). The incidences of lung adenomas and combined lung adenomas and carcinomas in females exceed the historical control ranges given by the performing laboratory, however the concurrent control group incidences of lung adenomas and combined lung adenomas and carcinomas in females also exceed these historical control ranges (Table 5b).

TABLE 3a. Valifenalate – Crl:CD-1(ICR)BR Mouse Study
(MRID No. 49807232)

Male Liver Tumor Rates[+] and Fisher's Exact Test and Exact Trend Test Results

	Dose (ppm)			
	0	150	850	5000
Adenomas (%) P =	7/50 (14) 0.00174**	2[a]/49 (4) 0.98336	14/49 (29) 0.06282	16/48 (33) 0.02117*
Carcinomas (%) P =	2/50 (4) 0.00369**	4/49 (8) 0.32920	4/49 (8) 0.32920	10[b]/48 (21) 0.01134*
Combined (%) P =	9/50 (18) 0.00008**	6/49 (12) 0.85973	16[c]/49 (33) 0.07368	23[d]/48 (48) 0.00150*

+Number of tumor bearing animals/Number of animals examined, excluding those that died before week 31.
[a] First adenoma observed at week 31 in the 150 ppm dose group.
[b] First carcinoma observed at week 72 in the 5000 ppm dose group.
[c] Two animals in the 850 ppm dose group had both an adenoma and a carcinoma.
[d] Three animals in the 5000 ppm dose group had both an adenoma and a carcinoma.

Note: Significance of trend denoted at control.
Significance of pair-wise comparison with control denoted at dose level.
If *, then $p < 0.05$. If **, then $p < 0.01$.

TABLE 3b. Valifenalate – Crl:CD-1(ICR)BR Mouse Study
(MRID No. 49807232) [a]

Historical control data for hepatocellular tumors in recent studies performed at the Eye Research Centre (Males)

Code number **Start date** **Study duration (weeks)**	**cdm094** **Jan-94** **78**	**cdm097** **Jul-94** **79**	**cdm105** **Sep-96** **78**	**cdm107** **Sep-97** **78**	**cdm108** **May-98** **78**	**cdm110** **Nov-98** **78**	**Total**	**Range of percentages***
Liver								
Hepatocellular adenoma								
Incidence	6	10	11	4	6	10	47	
Percentage*	11.5%	19.2%	21.2%	7.8%	12.0%	20.0%	15.31%	7.8 - 21.2
Hepatocellular carcinoma								
Incidence	2	3	1	1	1	4	12	
Percentage*	3.8%	5.8%	1.9%	2.0%	2.0%	8.0%	3.91%	1.9 - 8.0
Number of animals examined	52	52	52	51	50	50	307	

[a] Data from Table 3 on page 31 in the study report (MRID 49807232)

TABLE 4a. Valifenalate – Crl:CD-1(ICR)BR Mouse Study
(MRID No. 49807232)

Female Liver Tumor Rates[+] and Fisher's Exact Test and Exact Trend Test Results

	Dose (ppm)			
	0	150	850	5000
Adenomas (%) P =	0/48 (0) 0.0023**	0/48 (0) 1.0000	2/45 (4) 0.2314	5[a]/48 (10) 0.0280*
Carcinomas (%) P =	0/48 (0) 0.5079	1[b]/48 (2) 0.5000	0/45 (0) 1.0000	0/48 (0) 1.0000
Combined (%) P =	0/48 (0) 0.0068**	1/48 (2) 0.5000	2/45 (4) 0.2314	5/48 (10) 0.0280*

+Number of tumor bearing animals/Number of animals examined, excluding those that died before week 52.
[a] First adenoma observed at week 56 in the 5000 ppm dose group.
[b] First carcinoma observed at week 79 in the 150 ppm dose group.

Note: Significance of trend denoted at control.
Significance of pair-wise comparison with control denoted at dose level.
If *, then $p < 0.05$. If **, then $p < 0.01$.

TABLE 4b. Valifenalate – Crl:CD-1(ICR)BR Mouse Study
(MRID No. 49807232) [a]

Historical control data for hepatocellular tumors in recent studies performed at the Eye Research Centre (Females)

Code number	**cdm094**	**cdm097**	**cdm105**	**cdm107**	**cdm108**	**cdm110**	**Total**	**Range of**
Start date	**Jan-94**	**Jul-94**	**Sep-96**	**Sep-97**	**May-98**	**Nov-98**		**Percentages***
Study duration (weeks)	**78**	**80**	**78**	**78**	**78**	**78**		
Liver								
Hepatocellular adenoma								
Incidence	1	0	0	0	0	0	1	
Percentage*	1.9%	0.0%	0.0%	0.0%	0.0%	0.0%	0.33%	0.0 - 1.9
Hepatocellular carcinoma								
Incidence	0	0	0	0	0	0	0	
Percentage*	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.00%	0.0 - 0.0
Number of animals examined	52	52	52	51	50	50	307	

[a] Data from Table 4 on page 32 in the study report (MRID 49807232)

TABLE 5a. Valifenalate – Crl:CD-1(ICR)BR Mouse Study
(MRID No. 49807232)

Female Lung Bronchioalveolar Tumor Rates[+] and Fisher's Exact Test and Exact Trend Test Results

	Dose (ppm)			
	0	150	850	5000
Adenomas (%)	7/50 (14)	5/49 (10)	8[a]/50 (16)	13/49 (27)
P =	0.0175*	0.8118	0.5000	0.0961
Carcinomas (%)	1[b]/50 (2)	1/49 (2)	1/50 (2)	1/49 (2)
P =	0.5105	0.7475	0.7525	0.7475
Combined (%)	8/50 (16)	6/49 (12)	9/50 (18)	14/49 (29)
P =	0.0212*	0.7947	0.5000	0.1032

+Number of tumor bearing animals/Number of animals examined, excluding those that died before week 22.
[a] First adenoma observed at week 22 in the 850 ppm dose group.
[b] First carcinoma observed at week 68 in the control group.

Note: Significance of trend denoted at control.
Significance of pair-wise comparison with control denoted at dose level.
If *, then $p < 0.05$. If **, then $p < 0.01$.

TABLE 5b. Valifenalate – Crl:CD-1(ICR)BR Mouse Study
(MRID No. 49807232) [a]

Historical control data for selected tumors in recent studies performed at the Eye Research Centre (Females)

Code number	**cdm094**	**cdm097**	**cdm105**	**cdm107**	**cdm108**	**cdm110**	**Total**	**Range of**
Start date	**Jan-94**	**Jul-94**	**Sep-96**	**Sep-97**	**May-98**	**Nov-98**		**percentages***
Study duration (weeks)	**78**	**80**	**78**	**78**	**78**	**78**		
Lungs								
Bronchioloalveolar adenoma								
Incidence	10	3	5	8	4	3	33	
Percentage*	19.2%	5.8%	9.6%	15.7%	8.0%	6.0%	10.75%	5.8 - 19.2
Bronchioloalveolar carcinoma								
Incidence	2	1	0	1	1	1	6	
Percentage*	3.8%	1.9%	0.0%	2.0%	2.0%	2.0%	1.95%	0.0 - 3.8
Number of animals examined	52	52	52	51	50	50	307	

[a] Data from Table 6 on page 34 in the study report (MRID 49807232)

Non-Neoplastic Findings

Non-neoplastic findings included increased centrilobular and/or generalised hepatocyte hypertrophy as well as increased absolute and relative liver weights in both sexes at 850 ppm. There was also an increased incidence of macroscopic liver abnormalities including liver masses, pale areas, accentuated lobular patterns, and eosinophilic foci in both sexes at 850, while at the same dose level there was an increased incidence of centrilobular vacuolation in males only.

TABLE 6. Valifenalate: Non-neoplastic Findings in Mouse Carcinogenicity Study (MRID 49807232)

Group/sex		**1M**	**2M**	**3M**	**4M**	**1F**	**2F**	**3F**	**4F**
Dosage (ppm)		**0**	**150**	**850**	**5000**	**0**	**150**	**850**	**5000**
Centrilobular hepatocyte hypertrophy	**Total**	**21**	**34***	**26**	**8****	**8**	**9**	**12**	**25****
	Slight	19	21	18	5	8	8	12	22
	Moderate	2	13	8	2	0	1	0	3
	Marked	0	0	0	1	0	0	0	0
Generalised hepatocyte hypertrophy	**Total**	**3**	**6**	**13***	**29****	**2**	**2**	**7**	**5**
	Slight	3	5	10	18	2	2	6	5
	Moderate	0	1	3	11	0	0	1	0
Centrilobular hepatocyte vacuolation	**Total**	**11**	**14**	**33****	**32****	**2**	**8**	**8**	**2**
	Minimal	3	4	2	0	2	1	4	1
	Slight	7	7	20	11	0	7	4	0
	Moderate	1	3	11	20	0	0	0	1
	Marked	0	0	0	1	0	0	0	0
Increased hepatocytic cytoplasmic eosinophilia		**0**	**1**	**1**	**29****	**1**	**0**	**0**	**6**
Pigment in macrophages		**1**	**2**	**4**	**12****	**12**	**20**	**13**	**31****
Pigment in hepatocytes		**1**	**0**	**0**	**18****	**0**	**0**	**3**	**13****
Eosinophilic foci		**1**	**0**	**1**	**1**	**0**	**1**	**1**	**4**
Number of animal examined		**50**	**50**	**50**	**50**	**50**	**50**	**50**	**50**

a Data were obtained from Text-table 5 on page 33 of MRID 49807232.
* $p<0.05$; ** $p<0.01$ when compared to Group 1 (Fisher's Exact Test)

Adequacy of Dosing for Carcinogenicity Assessment

The doses used in the study were considered adequate and not excessive. There were no significant effects on mortality in either sex. Evidence of non-overt toxicity included liver effects such as increased absolute and relative liver weights, generalized hepatocyte hypertrophy, liver masses, pale areas, accentuated lobular patterns and eosinophilic foci in both sexes. Males had shown an

increase in absolute and relative liver weights at 150 ppm and at 850 ppm also showed an increased incidence of centrilobular vacuolation compared to control animals, however the vacuolation observed at 150 ppm is not considered to be adverse as the overall increase in incidence is equivocal and does not show statistical significance. This level of cellular response to exposure to valifenalate demonstrates a significant toxic challenge to the animals and the many different types of adverse effects noted at this level serve to demonstrate the level of both adaptive and adverse response to this challenge. At 5000 ppm, an even higher incidence of these adverse effects are observed in both sexes as well as effects in other organs such as an increase in absolute and relative kidney weights in females. Therefore, the doses utilized in this study can be considered adequate for evaluation of carcinogenic potential.

2. *Combined Chronic Toxicity & Carcinogenicity Study in Rats*

Citation: Webley L. (2004) IR5885 Combined carcinogenicity and toxicity study by dietary administration to Han Wistar rats for 104 weeks. Huntingdon Life Sciences Ltd. Woolley Road, Alconbury, PE28 4HS, England, unpublished report No. IGA008/032888. MRID 49807231.

In a 104-week combined chronic toxicity/carcinogenicity study, valifenalate was administered to Han Wistar rats (50/animals/sex/dose) at concentrations designed to achieve dosages of 0, 15, 150, or 1000 mg/kg/day. These animals comprised the Carcinogenicity phase of the study. A further 20 male and 20 female rats assigned to each group at study initiation were sacrificed after completion of 52 weeks of treatment, and comprised the Toxicity phase of the study.

Survival Analyses

. There were no statistically significant survival disparities among the dose groups in male or female rats in either phase of the study (Table 7).

TABLE 7. Valifenalate: Mortality and Survival in Combined Chronic Toxicity/Carcinogenicity Study in Rats. (MRID 49807231) [a]

Group/sex	1M	2M	3M	4M	1F	2F	3F	4F
Dosage (mg/kg bw/day)	**0**	**15**	**150**	**1000**	**0**	**15**	**150**	**1000**
Toxicity phase								
Group size	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**
Total number of death	1	1	0	0	0	0	1	3
% Survival	95	95	100	100	100	100	95	85
Carcinogenicity phase								
Group size	**50**	**50**	**50**	**50**	**50**	**50**	**50**	**50**
Total number of death	17	7	9	11	13	16	11	15
% Survival	66	86	82	78	74	68	78	70

Tumor Analyses

No treatment-related tumors were seen in male or female Han Wistar rats at any dose level.

Non-Neoplastic Findings

A slight increased incidence of yellow staining in the perigenital area was observed in females receiving 1000 mg/kg bw/day. Brown urine staining on the cage paper was observed in males and females at this dosage, up to Week 23.

Hematological investigations indicated persistently low hemoglobin concentration, erythrocyte counts and hematocrit in both sexes at 1000 mg/kg bw/day. Urinalysis investigations revealed some elevation in pH, ketones (in males), and urine volume (in females) treated at the two highest concentrations. Specific gravity and protein concentration, in general, were lower in females at 150 and 1000 mg/kg bw/day during Week 25, whereas an increase in males treated at 1000 mg/kg bw/day during weeks 25 and 51 was observed.

Elevated absolute and relative liver weights were observed in both carcinogenicity and toxicity phase animals treated at 1000 mg/kg bw/day. An increase in relative kidney weights was also observed in toxicity phase males treated at 1000 mg/kg bw/day. Histopathology examinations performed in animals killed after 52 weeks of treatment indicated an increased incidence of pelvic/papillary epithelial hyperplasia in the kidney of females and follicular cell hypertrophy in the thyroids of males at 1000 mg/kg bw/day, however follicular cell hypertrophy was not observed in carcinogenicity phase animals.

TABLE 8. Valifenalate: Male Non-Neoplastic Histopathological Findings in Combined Chronic Toxicity/Carcinogenicity Study in Rats. (MRID. 49807231)[a]

Group/sex		**1M**	**2M**	**3M**	**4M**
Dosage (mg/kg bw/day)		**0**	**15**	**150**	**1000**
Follicular cell hypertrophy	**Total**	**3**	**2**	**5**	**11***
	Slight	3	2	5	10
	Moderate	0	0	0	1
Number of animal examined		20	20	20	20

a Data were obtained from Text table 4 on page 40 of MRID 49807231.
* $p<0.05$ when compared to Group 1

TABLE 9. Valifenalate: Female Non-Neoplastic Histopathological Findings in Combined Chronic Toxicity/Carcinogenicity Study in Rats. (MRID. 49807231)[a]

Group/sex		**1F**	**2F**	**3F**	**4F**
Dosage (mg/kg bw/day)		**0**	**15**	**150**	**1000**
Pelvic/papillary epithelial hyperplasia	**Total**	**9**	**10**	**5**	**25****
	Slight	7	7	4	17
	Moderate	2	3	1	7
	Marked	0	0	0	1

Number of animal examined		50	50	50	50

a Data were obtained from Text table 5 on page 41 of MRID 49807231.
** p<0.05 when compared to Group 1

Adequacy of Dosing for Carcinogenicity Assessment

The doses used in the rat carcinogenicity study were considered adequate and not excessive to evaluate the carcinogenic potential of Valifenalate. There were no significant effects on survival in either sex and the animals were tested up to the limit dose (1000 mg/kg/day).

IV. TOXICOLOGY

1. *Metabolism*

In a series of metabolism studies in rats (MRID 49807204, 49807206), the data showed that both the metabolism and excretion of valifenalate following single or repeat oral administration of either 100 mg/kg/day or 1000 mg/kg/day is rapid with over 90% of the administered dose recovered within 48 h of administration from the excreta. At 72 h post-dose, feces accounts for the highest proportion of the dose with mean values of 86.82% and 50.71% recovered in male and female animals, respectively. However, bile is also an important route of elimination with a mean of 64.55% (males) and 48.68% (females) of the dose recovered in bile over 48 h following a dose of 100 mg/kg/day. The urine from bile duct-cannulated animals contained mean values of 12.84% (males) and 30.79% (females) of the dose at 48 h post-dose which was similar to that for non-cannulated animals. By 48 h post-dose feces samples contained a mean of 16.63% (males) and 15.55% (females) of the dose, and this presumably represented unabsorbed dose.

Following a single oral dose of [^{14}C-U-phenyl]-valifenalate at 100 mg/kg bw, concentrations of radiocarbon in whole blood increase rapidly to mean peak concentrations of 12.45 µg-equiv/g at 2 h post-dose in males and 9.42 µg-equiv/g at 1 h post-dose in females. T_{max} was reached in both sexes at approximately 0.1 hours (6 minutes) post dose for 100 mg/kg, with T_{max} increasing with dose up to 2 hours post dose for 1000 mg/kg. By 48 h post dose, concentrations began approaching the limit of quantification. The maximum concentration reached in whole blood following a single oral administration at 1000 mg/kg bw was at 2 h post-dose for both male and female animals (30.24 and 19.86 µg-equiv/g, respectively).

Following a single low dose of [^{14}C-U-phenyl]-valifenalate to male and female rats, the tissue containing the highest concentration of radiocarbon at C_{max} is the gastrointestinal tract. The only other tissues to contain a concentration greater than that associated with whole blood were liver and kidney. This pattern of distribution is also observed at the high-dose level and in animals that received repeated administrations of valifenalate. At 72 h post-dose, the carcass, including all organs and tissues, showed values of less than 0.22% in all cases.

Valifenalate is metabolized extensively and the qualitative metabolic profile was similar for all dosing regimens regardless of sex. However, there were quantitative differences in the amount of each metabolite, especially between the low and high doses. Identical metabolites were found both in urine and feces. Valifenalate acid (R2) was the main degradation product in feces, bile and urine for all administration doses at essentially the same proportion as unchanged parent valifenalate.

Five other metabolites were found, but did not reach 6% of the administered dose in the excreta. Among these, R3 and R4 were identified as RS-β-alanine, N-[(1-methylethoxy)carbonyl]-L-valyl-3-(2-hydroxy-4- chlorophenyl) and RS-β-alanine, N-[(1-methylethoxy)carbonyl]-L-valyl-3-(3-hydroxy-4- chlorophenyl), and R5 was identified as 3-amino-3-(4-chlorophenyl) propionic acid.

2. *Mutagenicity*

There is no concern for mutagenic activity with valifenalate. A battery of mutagenicity studies including, bacterial reverse mutation, in vitro mammalian cell gene mutation, in vitro mammalian chromosome aberration, and mouse erythrocyte micronucleus all produced negative results. A summary of the findings are presented below:

Test	**Study Information**	**Results**
Bacterial reverse mutation test OCSPP 870.5100	MRID 49807225 Valifenalate (98.9%) Acceptable/Guideline Concentrations up to 5000 µg/plate (with and without activation)	Negative. There was no evidence of induced reverse mutations with or without activation.
In vitro mammalian cell gene mutation test (mouse lymphoma assay) OCSPP 870.5375	MRID 49807229 Valifenalate (98.9%) Acceptable/Guideline Concentrations up to the limit of solubility and cytotoxicity (800 µg/mL)	Negative. There was no evidence of gene mutations with or without activation.
In vitro mammalian chromosome aberration test (CHO cells) OCSPP 870.5375	MRID 49807226 Valifenalate (98.9%) Acceptable/Guideline Tested up to cytotoxic concentrations (>200 µg/mL)	Negative. There was no significant induction of chromosomal aberrations.
Mammalian erythrocyte micronucleus-Mouse OCSPP 870.5395	MRID 49807230 Valifenalate (99.6%) Acceptable/Guideline Tested up to 2000 mg/kg (limit dose)	Negative. There was no significant increase in the frequency of micro-nucleated polychromatic erythrocytes.

3. *Structure Activity Relationship*

Valifenalate and its degradates were run through the DEREK system and there was one outcome of interest. The parent and 3 degradates contain a carbamate moiety and this moiety was flagged by DEREK for potential liver toxicity. A substructure search of all registered active ingredients (US) for the carbamate moiety came up with 23 hits. Of the 23 carbamate-containing structures, 18 had been reviewed by the CARC for potential carcinogenicity. Of the 18 CARC-reviewed structures, 6 had positive classifications as either "likely" or "probable" carcinogens (5) or "possible" carcinogens (1). All of the "likely/probable" chemicals were associated with liver tumors in mice, while the one classified as "possible" exhibited vascular tumors in mice. Rats exhibited liver, thyroid, uterine or bladder tumors in 4 of the 6 positively classified chemicals, while liver tumors were observed in 5 of the 6. The finding of liver tumors in mice in 5 other chemicals which share the carbamate chemical moiety lends support to the finding of mouse liver tumors in the valifenalate chemical database.

In the Alan Wood database there are 2 chemicals in the same chemical class as valifenalate (acylamino fungicides), benalaxyl and metalaxyl. CARC review indicated benalaxyl was positive for liver tumors in rats and mice and thyroid tumors in rats. Metalaxyl was negative for carcinogenicity.

4. *Subchronic and Chronic Toxicity*

13-Week Oral Toxicity Study in Mice

In a 13-week oral toxicity study (MRID 49807219), valifenalate ((98.9% a.i.,FCF/T/180-00 (ex ZI 068)) was administered to ten CD-1 mice/sex/dose in the diet at concentrations which were designed to achieve internal doses of 0, 15.3/14.7, 133.7/147.5 or 995/1144 mg/kg/day [M/F].

There were no mortalities and no clinical signs related to treatment observed in either sex as a result of exposure to valifenalate. Bodyweight gain was reduced in males at the high-dose and in females at the mid-dose, however overall bodyweight did not show significant deviation from controls. Observations over the course of treatment showed lower hematological factors compared to controls in males and females receiving the highest dose including lower hematocrit and hemoglobin concentrations, reduced mean cell hemoglobin and reduced cell volume. However, these changes were minimal and not associated with any histopathological changes in the animals.

Liver weights were unchanged with the exception of high dose males during the 13th week of treatment. Increased relative liver weights compared to controls were observed in males at the high-dose of 995 mg/kg bw/day. This was in addition to an increased incidence of centrilobular and periportal hepatocellular vacuolation due to a greater overall presence of fat in the liver. Fatty liver was observed in a high percentage of animals across all dose groups in both sexes (8/10 males vs 6/10 females), however this effect was also seen in a high percentage of control animals in both sexes (8/10 males vs 5/10 females) and was therefore not considered related to treatment. Females showed no treatment-related histopathological changes in the liver.

90-Day Oral Toxicity Study in Rats

In a 90-day oral toxicity study (MRID 49807220, valifenalate (98.9% a.i., FCF/T/180-00 (ex ZI

068)), was administered to male and female Han Wistar rats (ten/sex/dose) in the diet (7 days/week) at concentrations which were designed to achieve internal doses of 0, 7, 150 or 1000 mg/kg bw/day respectively. A further group of rats (five/sex/dose) were assigned to the control and high dose groups for a 4-week recovery period following the 13-week treatment period. The purpose of the recovery period was to assess the potential reversibility of any short-term or transient treatment-related effects observed during the 13-weeks of treatment.
There were no compound related effects on mortality, clinical signs, body weight, food consumption, hematology, clinical chemistry, organ weights, neuropathology, or gross and histologic pathology. There was a statistically significant increased incidence of distended caecum observed in males and females receiving 1000 mg/kg/day, however this was not associated with any histopathological changes in the animals and was not observed in test animals after the 4-week recovery period. Therefore, this was not considered to be adverse.

Carcinogenicity Study in Mice

In a 78-week oral carcinogenicity study (MRID 49807232), valifenalate was administered to Crl: CD-1™ (ICR) BR mice (50 mice/sex/group) at concentrations of 0, 150, 850 and 5000 ppm. Mean daily intakes were 0, 16.8, 97.2 and 657 mg/kg bw/day in males and 0, 21.6, 124 and 756 mg/kg bw/day in females, respectively. Parameters evaluated included, clinical condition, bodyweight, food consumption, hematology, organ weight, and macroscopic and microscopic pathology.

Mortality was not affected by treatment with the test item, as well as the appearance and behavior of treated animals. There were no treatment related effects on absolute bodyweight in either sex.

Examination of tail vein smears revealed no changes attributable to treatment. Absolute and relative liver weights were elevated in animals receiving 850 and 5000 ppm and in males at 150 ppm. Absolute and relative kidney weights were elevated in females receiving 5000 ppm. Macroscopic examination at necropsy revealed high incidence of liver masses, pale areas, and accentuated lobular pattern on the liver in males that received 850 and 5000 ppm, and an increased incidence of dark areas on the liver in females receiving 5000 ppm. An increased incidence of hepatocellular adenoma was observed in both sexes at 850 and 5000 ppm, and hepatocellular carcinoma in males receiving 5000 ppm.

Non-neoplastic histological changes in the liver included increased centrilobular and/or generalized hepatocyte hypertrophy in males receiving 150 ppm or above and in females receiving 850 ppm or above. The increased incidence in males at 150 ppm was not considered to be adverse as the majority of cases were slight and the overall number of slight cases observed is similar to the number of slight cases in control animals while the increased number of moderate cases does not display a dose-response. Other effects included increased cytoplasmic eosinophilia and increased pigment in macrophages and hepatocytes in animals receiving the highest dosage; increased eosinophilic foci and centrilobular vacuolation in males that received 850 ppm or above.

Combined Chronic Toxicity and Carcinogenicity Study in Rats

In a 104-week combined chronic toxicity/carcinogenicity study (MRID 49807231), valifenalate was administered to Han Wistar rats (50/animals/sex/group) at concentrations designed to achieve dosages of 0, 15, 150, or 1000 mg/kg bw/day for 104 weeks. These animals comprised the

Carcinogenicity phase of the study. A further 20 male and 20 female rats assigned to each group at study initiation were sacrificed after completion of 52 weeks of treatment, and comprised the Toxicity phase of the study.

Parameters evaluated included clinical condition, detailed physical and arena observations, sensory reactivity, grip strength, motor activity, bodyweight, food consumption, water consumption, ophthalmic examination, hematology, blood chemistry, urinalysis, organ weight, macropathology, and histopathology.

Mortality, neurotoxicity, ophthalmology and absolute bodyweight were unaffected by treatment. A slight increased incidence of yellow staining in the perigenital area was observed in females receiving 1000 mg/kg bw/day. Brown urine staining on the cage paper was observed in males and females at this dosage, up to Week 23.

Hematological investigations indicated persistently low hemoglobin concentration, erythrocyte counts and hematocrit in both sexes at 1000 mg/kg bw/day. Examination of tail vein smears taken from carcinogenicity-phase animals did not reveal any treatment-related change. There were no toxicological changes in the blood plasma. Urinalysis investigations revealed some elevation in pH, ketones (in males), and urine volume (in females) treated at the two highest concentrations. Specific gravity and protein concentration, in general, were lower in females at 150 and 1000 mg/kg bw/day during Week 25, whereas an increase in males treated at 1000 mg/kg bw/day during weeks 25 and 51 was observed. The appearance and composition of urine during weeks 77 and 103 were unaffected by treatment.

Elevated absolute and bodyweight-relative liver weights were observed in both carcinogenicity and toxicity animals treated at 1000 mg/kg bw/day. Bodyweight-relative higher kidney weights were also observed in Toxicity-phase males treated at 1000 mg/kg bw/day. Macroscopic examinations after 52 and 104 weeks of treatment did not reveal any findings related to treatment.

There were no neoplastic changes due to treatment with valifenalate.

Histopathology examinations performed in animals killed after 52 weeks of treatment indicated an increased incidence of pelvic/papillary epithelial hyperplasia in the kidney of females at 1000 mg/kg bw/day and follicular cell hypertrophy in the thyroids of males receiving 1000 mg/kg bw/day, however follicular cell hypertrophy was not observed in carcinogenicity phase animals.

5. *Mode of Action*

Introduction

The registrant (FMC Corporation) has submitted a mode of action analysis (MRID 49807234) that uses the International Programme on Chemical Safety (IPCS) framework and has also submitted a battery of mechanistic studies in support of its postulated mode of action for valifenalate-induced hepatocarcinogenesis in male and female mice. The registrant's proposed mode of action is presented below.

A. Postulated MOA for liver tumors in male and female mice

In terms of the mode of action for the observed liver tumors in mice, the registrant has postulated that the oral exposure of mice to valifenalate causes the co-activation of the nuclear receptors CAR, PXR and PPARα, which mediate the induction of replicative DNA synthesis and, ultimately, tumor development in the liver of CD-1 mice.

Key events

The registrant's postulated mode of action for the development of hepatocarcinogenicity in mice following exposure to valifenalate involves co-activation of the PPARα, CAR and PXR nuclear receptors and is defined by three key events in the temporal chain:

- Activation of nuclear hormone receptors, specifically CAR, PXR and PPARα (**Key Event #1**)
- Induction of CYP enzymes (**Associated Event**)
- Induction of replicative DNA synthesis (**Key Event #2**)
- Increased liver weights (**Associated Event**)
- Formation of hepatocellular carcinomas (**Key Event #3**)

These three key events are common to other chemicals which have been found to act through agonism of the nuclear receptors PPARα, CAR and PXR. The specific details of the biological response to exposure with valifenalate and the scientific evidence supporting the relation of those responses to these three key events is detailed below.

Data supporting Key Event #1: Activation of nuclear hormone receptors, specifically CAR, PXR and PPARα

In the position paper from the registrant (MRID 49807234), the proposed non-genotoxic MOA for the hepatocellular tumors observed in male and female mice involves the activation of the nuclear receptors CAR, PXR and PPARα. This activation leads to increased hepatocellular proliferation, which ultimately results in hepatocellular tumors.

In a 14-Day mechanistic feeding study in male CD-1 mice (Broich, 2015; MRID 49807233), animals were exposed to valifenalate in diet at dose levels of 0, 150, 1750 and 7000 ppm (0, 20.7, 249 and 1049.5 mg/kg/day) for 3 or 14 days. Phenobarbital was used as a positive control at 850 ppm (129.6 mg/kg/day. The study evaluated the following parameters: mortality, clinical signs, body weights and food consumption, absolute and relative organ weights, blood chemistry, histopathology, liver enzymes (cytochrome P450 mRNA levels and activity), oxidative stress parameters (TBARS; GSH), and cellular proliferation. The parameters relevant to the proposed MOA are reported below.

CAR, PXR and PPARα were all shown to be activated when exposed to high concentrations of valifenalate in the diet using CYP genes as surrogates for nuclear receptor activation. This activation was measured via the induction of mRNA gene expression for hepatic xenobiotic metabolizing enzymes Cyp1a1 (AhR), Cyp1a2 (AhR), Cyp2b10 (CAR) and Cyp3a11 (PXR) (Table 8).

Following exposure, Cyp1a1, Cyp1a2 and Cyp3a11 all showed dose-dependent increases in gene expression relative to vehicle controls beginning at a dose level of 249 mg/kg/day and showed marked increases in gene expression at a dose of 1049.5 mg/kg/day. Treatment with valifenalate was also shown to increase overall hepatocellular peroxisome proliferation beginning at a dose level of 249 mg/kg/day. Aryl hydrocarbon nuclear receptor (AhR) activation was also investigated but was shown to be unaffected by treatment with valifenalate as measured by mRNA expression for Cyp1a2 (Table 8). Gene expression data were generated in a separate 7 and 14-Day feeding study comparing wild-type and PPARα knockout C57BL/6 mice (Table 9).

TABLE 8. Valifenalate: CYP gene expression and enzyme activities [a]

CYP Gene Expression	**Control**	**20.7 mg/kg bw/day**	**249 mg/kg bw/day**	**1049.5 mg/kg bw/day**	**129.5 mg/kg/day Phenobarbitone**
Gene expression (fold changes)					
Cyp1a1	1.0	0.81	1.22	1.19	3.58
Cyp1a2	1.0	0.68	0.83	0.31	2.96
Cyp2b10	1.0	1.57	6.20	20.13	223.0
Cyp3a11	1.0	1.09	6.08	9.54	12.12
Enzyme Activity (%)					
MROD (Cyp 1a2)	100	108	126	118	312**
PROD (Cyp2b1)	100	79.5	165**	266**	1916**
6β-TOH (Cyp 3a)	100	93.1	150**	267**	420**
LA11OH (Cyp2e1)	100	101	206	384**	370**
LA12OH (Cyp4a1)	100	117	408*	1106**	214**
pCoA (Peroxisomal β-oxidation)	100	137	208**	308**	30**

[a] Data from page 203 of study MRID 49807233 and p 27 of report MRID 49807234
*p<0.05; ** p<0.01

TABLE 9. Valifenalate: mRNA expression as fold-differences from control values after 14 days of treatment in male WT and PPARα Knockout mice [a]

Gene	**WT**		**PPARα KO**	
	Control (0 ppm)	**Valifenalate (7000 ppm)**	**Control (0 ppm)**	**Valifenalate (7000 ppm)**
Cyp2b10	1.00	55.59 ± 14.6 [b]	1.00	48.09 ± 14.4 [b]
Cyp3a11	1.00	6.26 ± 1.7 [b]	1.00	8.49 ± 1.4 [b]
Cyp4a10	1.00	5.89 ± 2.0 [b]	1.00	68.8 ± 33.1 [b]
Cyp4a14	1.00	15.49 ± 6.2 [b]	1.00	151.59 ± 82.8 [b]
Acox1	1.00	2.06 ± 1.7 [b]	1.00	1.34 ± 0.2 [b]

Data from Table 10 on page 34 of the study report (MRID 49807218)
b p<0.001

Additional support of CAR/PXR and PPARα activation were observed in a study where a C57BL/6 (PPARα KO) strain of mouse was compared with wild-type C57BL/6. In both strains there was an increase in the liver: body-weight ratio but the relative liver weight increase in the wild-type was considerably greater than the knockout strain, consistent with activation of the PPARα receptor in the wild-type (Table 10). However the knockout strain did maintain a small increase in liver: body-weight ratio over the knockout vs. the control group, consistent with co-activation of other

receptors, subsequently confirmed as CAR/PXR. Secondly, the hepatic hypertrophic response to valifenalate was very much greater in the wild-type than the knockout strain. This is consistent with most of the hypertrophic responses being related to the PPARα activation, with only a relatively minor hypertrophic response expected through activation of CAR and PXR (Vardy 2015b; MRID 49807252).

Table 10. Valifenalate: Group Mean Body Weights, Weight Gain, and Absolute and Relative Liver Weights (g) [a]

Parameter	WT mice control	WT mice valifenalate (7000 ppm)	PPARα KO mice control (0 ppm)	PPARα KO mice valifenalate
Day 7				
Day 1 Body weight (g)	23.30 ± 1.04	22.95 ± 0.69 (-1.5%)	21.33 ± 1.00	22.21 ± 1.93 (+4.1%)
Terminal Body weight (g)	24.03 ± 1.50	24.85 ± 1.04 (+3.4%)	22.84 ± 0.91	23.32 ± 1.84 (+2.1%)
Body weight gain (g)	0.73 ± 0.88	1.90 ± 0.59 [b] (+159.2%)	1.51 ± 0.51	1.11 ± 0.57 (-26.9%)
Liver Weight (g)	1.29 ± 0.21	1.56 ± 0.17 [b] (+21.3%)	1.26 ± 0.08	1.37 ± 0.08 [b] (+9.1%)
Liver/Body weight ratio	5.34 ± 0.61	6.30 ± 0.66 [b] (+18%)	5.50 ± 0.18	5.90 ± 0.34 [b] (+7.2%)
Day 14				
Day 1 Body weight (g)	23.18 ± 0.65	22.28 ± 0.59 [b] (-3.9%)	21.71 ± 1.47	23.37 ± 1.74 [c] (+7.7%)
Terminal Body weight (g)	24.83 ± 1.11	24.42 ± 1.20 (-1.7%)	24.38 ± 1.55	24.80 ± 1.60 (+1.7%)
Body weight gain (g)	1.65 ± 1.01	2.14 ± 0.89 (+29.3%)	2.67 ± 1.37	1.43 ± 0.81 [c] (-46.4%)
Liver Weight (g)	1.32 ± 0.06	1.55 ± 0.15 [d] (+17%)	1.29 ± 0.08	1.45 ± 0.11 [b] (+12.4%)
Liver/Body weight ratio	5.33 ± 0.20	6.33 ± 0.36 [d] (+18.7%)	5.28 ± 0.15	5.83 ± 0.21 [d] (+10.4%)

Data from Tables 4 and 5 on Pages 20-21 of the study report (MRID 49807218)
a Values are Mean ± SD. Values in parenthesis are mean % control ± SD for the appropriate strain (n = 10 per group). A Student's t-test was performed on the results.
b Statistically different from control, $p<0.01$.
c Statistically different from control, $p<0.05$.
d Statistically different from control, $p<0.001$.

Further information, to strengthen the rationale of co-activation of PPARα/CAR/PXR derived from the knockout study, was analysis of gene expression which confirmed induction of mRNA of Acox1, Cyp2b10 and Cyp3a11 in the wild-type strain. The consequential enzyme induction profile was also characterized.

Peroxisomal ACox 1 mRNA (a marker of PPARα activation) was found to be reduced slightly in the valifenalate-treated knockout strain (1.3-fold relative to knockout negative control) relative to the wild type strain (1.8-fold relative to wild type negative control) confirming the presence of a relatively weak PPARα agonist. This relatively weak PPARα agonist activity was confirmed with higher 12-hydroxylauric acid (LAH) induction in the wild type (7.75 fold relative to negative control) compared to the knockout (4-fold relative to knockout negative control) (Table 11).

For Cyp2b10 mRNA (a marker of CAR activation), there was a rather weak, if any, increase in the valifenalate-treated wild type (56-fold increase relative to the wild type negative control) compared to the knockout strain (48-fold increase relative to the knockout negative control) indicative of activation of CAR of similar magnitude, in both the wild type and knockout strains. This was confirmed with pentoxyresorifin-*O*- dependylase (PROD) induction (a mixed function oxidase enzyme specific for Cyp2b10 activity) being similar in the wild type (6-fold increase relative to the wild type negative control) compared to the knockout (7-fold increase relative to the knockout vs. control) and was indicative of a relatively weak induction of Cyp2b10 (Table 11).

The induction of Cyp3a11 mRNA (a marker of PXR activation) in valifenalate-treated C57BL/6 mice was similar in the valifenalate treated wild type (6.3-fold increase relative to the wild type negative control) compared to the valifenalate-treated knockout strain (8.5-fold increase relative to the knockout negative control) and indicated activation of PXR of similar magnitude, in both wild type and knockout strains. Confirming this interpretation, there was also induction of Cyp3a11 enzyme activity, as monitored by benzyloxyquinoline-*O*-debenzylation (BQ) to the same extent in the wild type strain (2.4-fold increase relative to the wild type negative control) compared to the knockout strain (also 2.4-fold increase relative to the appropriate negative control value) (Table 11).

Table 11. Valifenalate: Biochemical Measurements after in WT and PPARα Knockout Mice-14 days[a]

Strain	WT		PPARα KO	
Treatment	**Control (0 ppm)**	**Valifenalate (7000 ppm)**	**Control (0 ppm)**	**Valifenalate (7000 ppm)**
PCoA nmol NAD+ reduced/min/mg protein	17.03 ± 3.36	34.49 ± 2.71 [b] (+102.5%)	11.71 ± 0.86	15.70 ± 1.76 [b] (+34.2%)
PROD pmols resorufin formed/min/mg protein	2.23 ± 0.58	13.45 ± 3.34 [b] (+503.5%)	2.70 ± 0.37	19.10 ± 2.59 [b] (+606.7%)
BQ nmols 7-OH quinolone formed / min / mg protein	2.06 ± 0.55	5.02 ± 0.43 [b] (+144.3%)	2.43 ± 0.33	5.85 ± 0.52 [b] (+140.6%)
LAH nmols 12-OH formed /10min / mg protein	2.74 ± 0.95	21.22 ± 3.12 [b] (+674.6%)	1.02 ± 0.14	4.09 ± 1.24 [b] (+300%)

[a] Data from Table on page 31 of the study report (MRID 49807218)
[b] $p<0.001$

<u>CARC conclusions on the data supporting Key Event 1</u>: CARC concluded that the surrogate data (induction of corresponding Cyp expression and enzyme activities) for Key Event 1 adequately support evidence of PPARα, CAR, and PXR co-activation.

Data supporting Key Event #2: Induction of replicative DNA synthesis

Evidence for the second key event, induction of replicative DNA synthesis, was obtained from BrdU incorporation studies. In one study (MRID 49807233), where CD-1 mice were fed valifenalate for 3 or 14 days, it was concluded that S-phase DNA synthesis was dose, and time-dependent, and was only present when mice were exposed to valifenalate at high doses near the carcinogenic dose-levels (Tables 12 and 13). Increased cell proliferation was not observed when valifenalate was administered to CD-1 mice at non-carcinogenic dose levels.

From the study in which C57BL/6 wild-type and PPARα KO strains were exposed to high levels of dietary valifenalate (MRID 49807218), it was clear that replicative DNA synthesis was increased 8-fold on day 7 for the wild-type (PPARα/CAR/PXR co-activated) but remained 5-fold elevated in the KO strain (only CAR/PXR co-activated), and was indeed confirmatory evidence that PPARα was not the only nuclear receptor involved in valifenalate-mediated hepatocarcinogenesis. As may be anticipated, by day 14 the initial wave of synthesis inducted by valifenalate, had subsided to about 3-fold and 2-fold for the wild-type and KO strains, respectively (Table 14).

This data is consistent with confirmation of the hypothesis that valifenalate activates multiple nuclear receptors (PPARα/CAR/PXR) and is a relatively weak agonist of PPARα. In addition, when PPARα was activated, it appeared that the degree of the hepatic hypertrophic response was greater than the hyperplastic response, whereas in contrast, with CAR/PXR activation, the

hypertrophic response generally appears less than the hyperplastic response.

Table 12. Replicative DNA Synthesis in CD-1 mice fed valifenalate in diet for 3 days

Group	Mean	SD	Min	Max
0 ppm IR5885	18	14	5	39
150 ppm IR5885	34	16	15	62
1750 ppm IR5885	65	43	19	143
7000 ppm IR5885	69	12	49	86
850 ppm Phenobarbitone	164	57	112	256

Data from Tables 3 on Pages 20-21 of the study report (MRID 49807233)

Table 13. Replicative DNA Synthesis in CD-1 mice fed valifenalate in diet for 14 days

Group	Mean	SD	Min	Max
0 ppm IR5885	15	9	5	25
150 ppm IR5885	37	17	10	55
1750 ppm IR5885	51	31	8	101
7000 ppm IR5885	60	30	13	97
850 ppm Phenobarbitone	80	28	42	124

Data from Tables 4 on Pages 34 of the study report (MRID 49807233)

Table 14. Valifenalate: S-Phase DNA replication [a]

Strain	Day	Control 0 ppm	Valifenalate 7000 ppm
WT	7	1.90 ± 0.30	15.46 ± 2.85 [b, c] (+715.4%)
PPARα KO		0.79 ± 0.15	4.24 ± 2.18 [c] (+436.8%)
WT	14	2.66 ± 1.08	9.42 ± 3.57 [c] (+254.7%)
PPARα KO		1.68 ± 0.67	3.14 ± 0.93 [c] (+86.9%)

Data from Table 7 on page 27 of the study report (MRID 49807218)
a Values are Mean ± SD. Values in parenthesis are mean % control ± SD for the appropriate strain (n = 10 per group). A Student's t-test was performed on the result.
b n = 9 as mouse #29 was considered an outlier.
c Statistically different from control, $p<0.001$.

CARC concurs that the data is consistent with the hypothesis that valifenalate activates multiple nuclear receptors (PPARα/CAR/PXR) and results in a short (burst) proliferative response (replicative DNA synthesis) supporting key event 2.

Data Supporting Key Event #3: Formation of Hepatocellular Adenomas/Carcinomas

For valifenalate, as shown in the mouse carcinogenicity study, hepatocellular adenomas and carcinomas were reported at dietary incorporation levels of 850 and 5000 ppm in male CD-1 mice. In contrast in the same study, incorporation of valifenalate to diet of male CD-1 mice at the level of 150 ppm did not produce hepatocellular adenomas or carcinomas.

In Broich, 2015 the overall levels of CAR, PXR, and PPARα activation were measured as well as the level of hepatocellular proliferation as measured by both BrdU nuclear incorporation as well as histopathological examination. The results of this study showed that while at least some portion of the overall observed hepatocellular hypertrophy is due to the co-activation of CAR and PXR, none of the receptors were significantly activated (as measured through Cyp450 subfamily mRNA expression) at doses less than 1750 ppm. At a dose level of 150 ppm, there was no significant increase in either gene expression or enzyme activity as compared to vehicle controls. This concurs with the observations of both increases in absolute and relative liver weight as well as hepatocellular hypertrophy, both of which only begin to occur at 1750 ppm.

Therefore, it would seem that dose levels which are insufficient to cause significant activation of the CAR, PXR and PPARα receptors (Event 1) prevent progression to increases in S-Phase DNA synthesis (Event 2) and therefore do not result in the formation of hepatocellular adenomas or carcinomas (Event 3).

CARC agrees with the evidence to support that the tumor observed at the high dose were treatment related in male and female mice.

Temporal Association

The first 2 Key Events in mice upon exposure to valifenalate (activation of nuclear receptors followed by an increase in replicative DNA synthesis), while consecutive, do occur in relatively quick succession in biological terms. As Key Event 1 is measured in the available studies through the indirect means of gene and enzyme induction, making an absolute differentiation in the time course between receptor activation and cellular proliferation is not possible with current information. However, the evidence provided in Broich, 2015 shows at least that replicative DNA synthesis does not take place independently at lower doses from those also shown to cause nuclear receptor activation.

The available data, which measure both receptor activation and cellular proliferation at the same time points across both studies, in this case 3, 7, and 14 days show that both key events are occurring at the same time-scales in dosed animals. It is important to note that while measurements of S-Phase DNA synthesis show the effect in real-time, as it is occurring since it is measured using direct nuclear incorporation of a signaling agent, measurements of nuclear receptor activation only show the effect occurring after-the-fact as there is a significant comparative delay between receptor

activation and induction of relevant gene expression.

The temporal association between this second key event, increased hepatocellular proliferation, and the third key event, increased hepatocellular carcinomas, is more clearly defined from the long-term studies on valifenalate. This sequence of events is consistent with the database available from the 78-week carcinogenicity study with valifenalate in CD-1 mice. Male CD-1 mice are more severely affected than female mice, with a greater number of foci developing towards adenoma and carcinoma in males. In female mice, carcinomas did not develop, and consistent with this, the incidence of foci at the highest dose level was greater than in the males, and a relatively small but significantly increased incidence of adenomas was reported in females (Webley 2004b).

In summary the evidence generated for valifenalate in CD-1 mice is consistent with the temporal association of a mode of action with three consecutive, key events and this information is summarized in Table 14 below. The molecular initiating event is the co-activation of multiple nuclear receptors, CAR/PXR/PPARα, and as a direct consequence, the associated induction of gene expression and enzyme activity of Cyp2b10, Cyp3a11 and Cyp4a. The second key event, increased hepatocellular proliferation, is also initiated in CD-1 mice exposed to valifenalate, on a time scale not dissimilar to the appearance of induction of the hepatic metabolizing enzymes. The final key event is the longer-term formation of carcinomas via the development of altered, hyperplastic, hepatic, foci and the subsequent development of benign and, ultimately, malignant hepatocellular neoplasms. This is consistent with information from the 78-week carcinogenicity study in male and female CD-1 mice.

Table 15. Valifenalate: Temporal Association Chart

		Time				
	Dose ppm (mg/kg bw/day)	**Key Event 1 Initiating Event Activation of CAR/PXR/PPARα**	**Key Event 2 Increased replicative DNA synthesis**	**Associated event: Increased hepatocellular hypertrophy**	**Key Event 3 Formation of Carcinoma/ Adenoma*** (see footnote)	**Reference**
		Measured indirectly from Day 7	*Measured from Day 3*	*Measured from Day 3 to 90*	*Key event: Measured at 78 weeks*	
Dose	110 (15.3)			- in CD-1 strain of mouse 90 days		Webley et al (2002)
	150 (20.7)	- day 14 in male CD-1 strain of mouse	- day 3 and day 14 in CD-1 strain of mouse	- in CD-1 strain of mouse at 3 & 14 days		Broich (2015)
	150 (16.8)			- in CD-1 strain of mouse at 78 weeks	- week 78 in CD-1 strain of mouse	Webley L (2004b)
	850 (97.2)			+ in CD-1 strain of mouse at 78 weeks	+ week 78 in CD-1 strain of mouse	Webley L (2004b)
	900 (133.7)			+ in CD-1 strain of mouse at 90 day		Webley et al (2002)
	1750 (249)	+ day 14 in male CD-1 strain of mouse	++ day 3 in CD-1 strain of mouse + day 14 in CD-1 strain of mouse	+ in CD-1 strain of mouse at 3 and 14 days		Broich (2015)
	5000 (657)			+ in CD-1 strain of mouse at 78 weeks	++ week 78 in CD-1 strain of mouse	Webley L (2004b)
	7000 (1049.5)		++ day 3 in CD-1 strain of mouse +(+) day 14 in CD-1 strain of mouse	+ in CD-1 strain of mouse at 3 & 14 days		Broich (2015)
	7000 (995)			+ in CD-1 strain of mouse at 90 days		Webley *et al* (2002)
	7000 (1050)	++ day 14 in male CD-1 strain of mouse	+ day 14 in CD-1 strain of mouse			Vardy A (2015a)
	7000 (1324-1636)	++ day 7 in male C57BL/6 and C57BL/6 (PPARα KO) strains of mouse	++ day 7 in C57BL/6 strain of mouse + day 7 in C57BL/6 (PPARα KO) strain of mouse	+ in CD-1 strain of mouse at 7 & 14 days		Vardy A (2015b)

Data from Table 3 on page 25 of the MoA white paper (MRID 40907234)

CARC agreed that there was sufficient evidence of temporal and dose concordance for the key events leading to the formation of hepatocellular adenomas/carcinomas in mice**.**

Strength, Consistency and Specificity of Association of Tumor Response with Key Events

The current carcinogenicity database for valifenalate includes two regulatory carcinogenicity studies (mouse, rat) as well as four regulatory mutagenicity studies and four additional mechanistic studies aimed at providing support for the proposed mechanism of action. In the mouse carcinogenicity study statistically increased incidences of both hepatocellular adenomas and carcinomas was reported in males while only hepatocellular adenomas were increased in females. In the parallel rat carcinogenicity study there were no neoplastic findings.

None of the other adverse effects reported in the mouse studies were inconsistent with the proposed mode of action for valifenalate-induced, receptor mediated carcinogenicity in mice. Another nuclear receptor, AhR, is not activated by valifenalate in CD-1 male mice (Broich, 2015) and the registrant suggests that this information is consistent with the known structure-activity relationships associated with this receptor, where agonists tend to be relatively large, planar, aromatic structures.

Four mechanistic studies were designed to support the hypothesis that the tumors induced by valifenalate occur via a mechanism mediated by activation of three nuclear hormone receptors, namely CAR/PXR/PPARα. In terms of dose-response relationship for valifenalate-induced carcinogenicity in mice, evidence from the short-term studies indicates that there is essentially no activation of CAR, PXR or PPARα at the lower dose level of 150 ppm (20.7 mg/kg/day) (Broich, 2015). As activation of these receptors is considered as the initiating event for valifenalate-induced carcinogenicity, it follows that subsequent key events should not occur *in vivo*.

This is supported by the outcome of the long-term carcinogenicity study where no tumors were reported at this dose level (150ppm). In short-term studies, as the exposure to valifenalate is increased and CAR/PXR/PPARα become co-activated, replicative DNA synthesis, the second key event, becomes measurable from as early as day 3 (Broich 2015). The registrant notes that this is the normal time period after initiation of dosing shown by the group of chemicals known to activate these receptors. The long-term consequences of this increase in hepatocellular proliferation is the development of altered, hyperplastic, hepatic foci, and the subsequent development of benign and, ultimately, malignant hepatocellular neoplasms. The incidence of these effects in male mice although somewhat increased at the intermediate dose-level of 850ppm, is markedly increased as exposure is increased to 5000ppm, the highest dose-level tested in the carcinogenicity study. This is consistent with the analysis of gene expression and enzyme induction where these parameters were higher at the top dose than at the intermediate dose level.

Finally, similar short-term studies with the C57BL/6 PPARα KO strain of mouse led to a reduction in PPARα activation. However, CAR/PXR could still be activated in the PPARα knockout strain of mouse, and hence a modified initiating event (i.e. activation of CAR/PXR in isolation) could occur in this transgenic strain, that could still lead to hepatocellular proliferation albeit to a reduced extent (Vardy 2015).

Therefore, all of the data gathered within the current carcinogenicity database are consistent with the three key events proposed for the mode of action of tumor formation in mice. Information from

the knockout study, in conjunction with other considerations of potential alternative mechanisms, provides a high degree of confidence relating to the correlation of the tumorigenic response to the hypothesized mode of action.

Biological Plausibility and Coherence

The registrant suggests that the hypothesized mode of action is not unique but has been, and continues to be, a well- studied area of investigative toxicology. Other chemical entities, of which sulfoxaflor is a recent example, have been described as causing an increased incidence of hepatocellular adenomas and carcinomas in rats and/or mice through activation of one or more of these nuclear hormone receptors (EFSA, 2014). In some instances, where the nuclear receptor activation is predominantly restricted to just the PPARα receptor, it has been shown using PPARα knockout mice that the second key event, increased hepatocellular proliferation, cannot proceed in the absence of activation of the receptor. Similarly, with other chemistries, where nuclear receptor activation is predominantly restricted to the CAR/PXR receptors, it has been shown using CAR/PXR knockout mice that the second key event, increased hepatocellular proliferation, cannot proceed in the absence of activation of the receptor. These studies have provided strong evidence that an initiating event for the formation of hepatocellular adenomas and carcinomas in rodents may be the activation of one or more of these nuclear hormone receptors.

Other detailed studies on later aspects of the development of rodent hepatic neoplasia have shown a direct correlation between the induction of hepatocyte DNA synthesis with the development of altered, hyperplastic, hepatic foci, followed by the development of benign and, ultimately, malignant hepatocellular neoplasms (Holsapple et al 2006; Klaunig et al 2004, Cohen 2010, Elcombe et al 2014). For valifenalate, the second key event has therefore been considered as increased replicative DNA synthesis which, if maintained over time, may ultimately lead to the final key step which is formation of malignant hepatocellular neoplasms. Studies with a range of different molecules, generally in hepatocytes from different species, have shown that although this increased replicative DNA synthesis may occur in rodents, it does not occur, or at least occurs to a much lesser degree, in humans.

Other Modes of Action

Other modes of action have been associated with rodent hepatic carcinogenesis (Cohen, 2010). Such mechanisms can be subdivided into those that involve a direct reaction with DNA and those that do not. Examples of those mechanisms devoid of such DNA-reactivity include cytotoxicity, estrogen-mediated, and receptor mediated mechanisms, such as those involving AhR, CAR, PXR and PPARα.

Direct reactivity with DNA: It has been clearly established from a battery of guideline *in vitro* and *in vivo* studies that valifenalate is not genotoxic and hence such a mode of action is unlikely.

AhR-mediated carcinogenesis: Activation of AhR can lead to the induction of enzymes that activate aryl hydrocarbons by forming epoxides that are DNA reactive, leading to tumor development. For valifenalate, an AhR-mediated mode of action for formation of adenomas and carcinomas in CD-1 mice is unlikely since neither gene expression nor enzyme activity of hepatic Cyp1a was induced in CD-1 mice exposed to valifenalate at any dose level. Therefore AhR is not

activated at dose levels where valifenalate induces hepatocellular carcinomas, and does not induce these effects, in male CD-1 mice (Broich, 2015).

Estrogen-mediated: Although estrogens have a receptor-mediated mode of action that includes hepatocellular proliferation, it has been proposed that this may be due to the formation of DNA adducts and increased cell proliferation (Cohen, 2010). There is no structural similarity between valifenalate and estrogen that might suggest a similar mode of action of hepatocarcinogenesis and there was no evidence of estrogenic activity in the guideline two-generation toxicity study in the rat.

Cytotoxicity-mediated: Several guideline oral mouse studies have been conducted using dietary incorporation of valifenalate. This includes some short-term studies conducted at very high levels of valifenalate exposure, up to about 1500 mg/kg in some studies/strains of mouse. In most studies, ALP, AST and ALT were measured as markers of hepatic necrosis. In these studies on valifenalate in the target species, i.e. mouse, there was no evidence of any significant perturbation that might indicate hepatic necrosis or cytotoxicity from either histopathology or clinical biochemistry at any of the dose levels to which mice were exposed. Overall, the evidence in the mouse does not support a mechanism involving sustained cytotoxicity / repair / proliferation eventually leading to the formation of hepatocellular carcinomas. With respect to cytotoxicity as an alternative mechanism for carcinogenicity, the information from rat, mouse and dog studies is fairly consistent with the hypothesized mode of action and suggests that cytotoxicity is unlikely to play a role in the carcinogenicity of valifenalate in CD-1 mice.

Uncertainties, Inconsistencies and Data Gaps

There are other chemical entities that have been described as causing an increased incidence of hepatocellular adenomas and carcinomas in rats or mice through activation of these hormone receptors. In some instances, where the nuclear receptor activation is more prominently restricted to just the PPARα receptor, it has been shown using PPARα knockout mice that the second key event, increased hepatocellular proliferation, cannot proceed (Corton et al 2014). Similarly with other chemistries, where nuclear receptor activation is predominately restricted to the CAR/PXR receptor, it has been shown using CAR/PXR knockout mice that the second key event, increased hepatocellular proliferation, cannot proceed (LeBaron et al., 2014).

There are other substances where all three receptors CAR/PXR/PPARα are co-activated, as with valifenalate, but as yet there is no feasible ‘triple’ knockout mouse strain available to clearly show that the second key event cannot proceed. However, there is good evidence, with respect to the mode of action, from dose-response relationships for valifenalate in male CD-1 mice that in the absence of the initiating event (activation of CAR/PXR/PPARα), the second key event, induction of replicative DNA synthesis, does not occur. Investigative studies with valifenalate in C57BL/6 and C57BL/6 (PPARα KO) strains have indicated that replicative DNA synthesis is significantly lower in the KO strain than in the wild-type, as might be expected when CAR/PXR may still be activated. It is considered that these data in conjunction with the lack of evidence for an alternative mode of action for the formation of the hepatocellular carcinomas serve to lend further support for this mode of action for valifenalate-induced liver tumors in CD-1 mice.

A comparison of *in vivo* and *in vitro* studies in CD-1 mice suggests that metabolic activation of

valifenalate may be necessary for receptor activation. In hepatocyte cultures, metabolism of valifenalate may not occur in sufficient quantities for activation of CAR/PXR/PPARα, therefore precluding induction of gene expression and enzyme induction and the subsequent key event, increased S-phase. In such a situation there is little value in using human hepatocyte studies to identify if the same receptors are activated and, in addition if the second key step, induced replicative DNA synthesis, occurs.

Human use of phenobarbital offers valuable insight, on account of the extensive epidemiological data available from its clinical use, finding no association between phenobarbital use and human cancer. The mode of action for phenobarbital-like P450 inducers was determined to be unlikely in humans after kinetic and dynamic factors were considered (Holsapple et al 2006, Elcombe et al 2014). The weight of evidence from this assessment indicates that valifenalate is such a phenobarbital-like P450 inducer as shown in comparison to valifenalate where phenobarbital was used as a positive control (Broich, 2015), albeit perhaps one with a somewhat increased induction of PPARα. However, PPARα induced rodent hepatocarcinogenesis has also been considered as not relevant to humans, on a quantitative basis.

Human Relevance

A phenobarbital-type mode of action has been identified, albeit with perhaps a slightly greater contribution of the nuclear receptor PPARα, with valifenalate. Therefore relevance to humans, of the valifenalate-induced hepatic tumors in mice, has been considered within the context of valifenalate co-activating CAR/PXR and PPARα. For clarity, the relevance to humans of CAR/PXR activation has been considered independently from the relevance to humans of activation of the PPARα receptor. Alternative modes of action, such as the direct reactivity of valifenalate towards DNA, valifenalate-induced cytotoxicity and/or hormonal perturbation, have also been considered.

1. The relevance to humans of valifenalate-mediated activation of CAR/PXR

Phenobarbital is typical of rodent hepatocarcinogens that induce tumors by a non-genotoxic mechanism, critically involving liver hyperplasia (Williams, 1997). Phenobarbital, like valifenalate, is neither cytotoxic or genotoxic (Elcombe et al. 2014). Induction of some Cyp enzymes, particularly of the Cyp2b family, are considered diagnostic for phenobarbital and are a consequence of activation of nuclear receptors, particularly CAR but also PXR and to a lesser extent PPARα. Phenobarbital also induces Cyp enzymes in human liver, although there are reports that it may act more through PXR than through CAR (Elcombe et al. 2014). It is this activation of CAR that is generally viewed as the first key event of phenobarbital-mediated hepatic tumors in rodents, and this event has been reported to occur in both rodents and humans.

There are some human studies that may shed light on the second key step, the increased replicative DNA synthesis. It has been reported using human hepatocytes that unlike rodent hepatocytes, these human cells are refractory to increased replicative DNA synthesis when exposed to phenobarbital (Parzefall et al., 1991; Hasmall and Roberts, 1999, Elcombe, 2014). Perhaps more importantly, there are clinical data on phenobarbital, where the substance has been used in patients for many years, exposed to plasma concentrations similar to those following a carcinogenic dose in rodents, and yet there is no evidence of a hepatocarcinogenic effect (Elcombe et al. 2014). Taken together

this evidence indicates that, at least on a quantitative basis, taking into account kinetic and dynamic factors, that the phenobarbital-mediated (CAR/PXR-mediated) hepatocarcinogenicity, expressed in rodents, is not likely to be expressed in humans.

An *in vitro* study (MRID 49807228), hereafter referred to as Vardy, 2015c was designed to directly investigate the potential of valifenalate to activate CAR/PXR and/or PPARα nuclear hormone receptors and stimulate cell proliferation, in isolated hepatocytes prepared from male CD-1 mice. Phenobarbital and Wy-14,643 were included as positive controls An initial cytotoxicity assay (assessing ATP levels) was performed with valifenalate. Depletion of ATP levels, and therefore cytotoxicity, was measured at 300 μM making this a suitable top concentration to be assessed in the main enzyme induction and proliferation study. Neither phenobarbital nor valifenalate had any impact on replicative DNA synthesis in the male CD-1 mouse hepatocytes in vitro. Phenobarbital has been reported to show a weak effect on mouse hepatocyte proliferation in vitro. However, Wy-14,643 increased replicative DNA synthesis by a maximum of 1.7-fold, and epidermal growth factor (EGF) also produced a robust response, demonstrating that the test system could respond to a proliferative stimulus.

In male CD-1 mouse hepatocytes, phenobarbital induced Cyp2b10 as measured by Taqman® mRNA analysis and PROD activity, but had little impact on the Cyp4a or peroxisome proliferation markers. Conversely, Wy-14,643 induced Cyp4a and peroxisome proliferation as measured by Taqman® mRNA analysis (Cyp4a10, Cyp4a14 and Acox1), 12- OH LA formation and PCoA oxidation. Valifenalate had essentially no impact on any of the biochemical markers assessed. It may be reasonable to consider that metabolism of valifenalate is likely to be a key factor in the activation of CAR/PXR and PPARα and that the hepatocyte culture system is incapable of producing the quantity(s) of the metabolite(s) necessary to co-activate CAR/PXR and PPARα. Evidence for this scenario comes from a comparison of the valifenalate-induced induction of the associated mRNAs and Cyp isozymes induced *in vivo*, but are absent, in this mouse (CD-1 strain) hepatocyte culture system, *in vitro*. Unfortunately these factors preclude a study to define a valifenalate-specific lack of induction of replicative DNA synthesis in human hepatocytes. These findings are summarized in Tables 14, 15 and 16.

Table 14. Valifenalate: ATP assay results for PB, Wy-14,643, and valifenalate.

Test item and concentration	ATP content (luminescence units) [a]
Vehicle control (0.1% [v/v] DMSO)	436918 ± 47416
PB 100 µM	405658 ± 42447
PB 1000 µM	432136 ± 27057
Wy-14,643 50 µM	475430 ± 45627
Wy-14,643 100 µM	496961 ± 76154
Valifenalate 10 µM	461539 ± 35657
Valifenalate 30 µM	440125 ± 13828
Valifenalate 100 µM	435432 ± 23177
Valifenalate 300 µM	116405 ± 4827 [b] (-73.4%)

Data from Table 3 on page 15 in the study report (MRID 49807228)

a Values are mean ± standard deviation; values in parenthesis are mean % control ± standard deviation; n = 6 per group. A Student's t-test (2-sided) was performed on the results.

b Statistically different from control, p<0.001.

Table 15. Valifenalate: Biochemical measurements for CD-1 mouse primary hepatocytes.

Test item and concentration	PROD (pmol resorufin/min/mg)[a]	LAH nmols 12-OH formed/60min/ mg protein[a]	PCoA nmol NAD+ reduced/min/mg protein[a]
Vehicle control (0.1% [v/v] DMSO)	9.49 ± 1.70	0.83 ± 0.16	0.25 ± 0.04
PB 100 µM	17.18 ± 1.70 [b] (+81.1%)	0.98 ± 0.25	0.53 ± 0.18
PB 1000 µM	31.79 ± 5.59 [b] (+235.2%)	1.57 ± 0.82	0.73 [c] (+191%)
Wy-14,643 50 µM	8.75 ± 0.72	6.71 ± 0.31 [d] (+711.3%)	1.23 ± 0.23 [b] (+390.6%)
Wy-14,643 100 µM	10.45 ± 1.38	5.74 ± 1.28 [d] (+594%)	1.36 ± 0.34 [b] (+442%)
Valifenalate 10 µM	13.79 ± 5.61	0.91 [c] (+9.9%)	0.51 [c] (+105%)
Valifenalate 30 µM	11.43 ± 0.82	0.80 ± 0.16	0.48 ± 0.21
Valifenalate 100 µM	12.85 ± 1.74	0.72 ± 0.08	0.40 ± 0.04 [b] (+60.6%)
Valifenalate 300 µM	9.15 ± 1.08	0.81 ± 0.10	0.37 ± 0.18

Data from Table 4 on page 17 of the study report (MRID 49807228)

a Values are mean ± standard deviation; values in parenthesis are mean % control ± standard deviation; n = 3 per group, except as noted. A Student's t-test (2-sided) was performed on the results.

b Statistically different from control, p<0.01.

c n = 2

d Statistically different from control, p<0.001.

Table 16. Valifenalate: Taqman analysis of mouse Cyp2b10, Cyp4a10, Cyp4a14, and Acox1 mRNA from CD-1 mouse hepatocyte

Test item and concentration	Cyp2b10 mRNA[a]	Cyp4a10 mRNA[a]	Cyp4a14 mRNA[a]	Acox1[a]
Vehicle control (0.1% [v/v] DMSO)	1.00	1.00	1.00	1.00
PB 100 μM	3.77 ± 0.9 [b]	1.51 ± 0.3 [c]	1.81 ± 0.5 [c]	0.84 ± 0.1
PB 1000 μM	9.11 ± 5.2	2.29 ± 0.2 [d]	3.70 ± 0.4 [d]	0.83 ± 0.1
Wy-14,643 50 μM	2.73 ± 0.4 [b]	391.8 ± 90.4 [b]	762.31 ± 138.7 [d]	2.07 ± 0.4 [c]
Wy-14,643 100 μM	2.96 ± 0.7 [c]	297.84 ± 24.6 [d]	643.0 ± 18.1 [d]	2.23 ± 0.2 [b]
Valifenalate 10 μM	0.61 ± 0.2	1.08 ± 0.3	2.32 ± 0.6 [c]	0.74 ± 0.1
Valifenalate 30 μM	0.70 ± 0.2	1.39 ± 0.1 [c]	3.01 ± 0.4 [d]	0.76 ± 0.1 [c]
Valifenalate 100 μM	0.82 ± 0.3	1.29 ± 0.2	1.61 ± 0.5	0.79 ± 0.1
Valifenalate 300 μM	0.54 ± 0.1 [c]	1.01 ± 0.2	0.54 ± 0.3	1.07 ± 0.1

Data from Table 5 on page 20 of the study report (MRID 49807228)

a Values are mean ± standard deviation; n = 3 per group. Mouse β-actin was employed as the internal control. A Student's t-test was performed on the results.

b Statistically different from control, $p<0.01$.

c Statistically different from control, $p<0.05$.

d Statistically different from control, $p<0.001$.

Despite the technical difficulties in assessing replicative DNA synthesis with valifenalate in cultured hepatocytes (Vardy, 2015c), there is much information within the open literature that clearly identifies the relevance to humans of a phenobarbital-type mode of action in rodents, in the absence of other alternative mechanisms known to have potential relevance to humans.

2. The relevance to humans of valifenalate-mediated activation of PPARα

With valifenalate, as with many other substances, the finding of hepatic peroxisome proliferation is highly specific, and indicates that PPARα activation played a role in the initial stages of this mode of action. However, well-established indirect measures of PPARα activation in both the CD-1, C57BL/6, and C57BL/6 (PPAR knockout) strains of mouse have been reported.

The PPARα mode of action is a well-established, and accepted, mode of action for the induction of liver tumors in rodents, and the carcinogenic non-relevance to humans is also scientifically well established (Klaunig et al., 2003). The data available for valifenalate provide a high level of confidence that this mode of action is operative in male CD-1 and C57BL/6 strains of mouse, in addition to activation of CAR/PXR.

The human relevance of the PPARα mode of action as a whole may not entirely be excluded simply on the basis of fundamental qualitative differences in key events between animals and humans. Indeed the basis for the use of fibrates as hypolipidemic agents is the activation of PPARα in order to produce some of the desired features of the pleiotropic responses shown by these

chemicals, namely their hypolipidemic effects. Therefore, in the absence of further species-specific mechanistic data, it is relevant to consider a quantitative difference in kinetic or dynamic factors between animals and humans, rather than qualitative species difference in response for this mode of action.

For PPARα agonists, the question of human relevance was the subject of a thorough review (Klaunig et al., 2003). The authors concluded that after taking kinetic and dynamic factors into consideration, the overall weight of evidence suggested that the rodent mode of action for PPARα activator-induced liver tumors was not likely to occur in humans. In 2010 a group of experts, aware of the increased understanding of the relationships between PPARα activation and liver cancer formation, critically re-evaluated the state of the science for the induction of liver tumors in rodents (Corton et al 2014). The key events of the mode of action for PPARα, and the ultimate conclusions arrived at by this workshop remained consistent with the earlier publication of Klaunig et al in 2003 i.e. PPARα-activation in humans does not result in increased liver tumors.

The weight of evidence indicates that valifenalate acts as a PPARα agonist and that after taking kinetic and dynamic factors into consideration, any hepatocellular carcinomas developed through activation of this nuclear hormone receptor by valifenalate in mice, is not likely to occur in humans.

3. The relevance to humans of valifenalate-mediated activation of CAR/PXR/PPARα

The evidence indicates that valifenalate acts as a co-activator of CAR/PXR and PPARα and, after taking kinetic and dynamic factors into account, any hepatocellular carcinomas developed through activation of these nuclear hormone receptors by valifenalate in mice, is not likely to occur in humans.

CARC conclusion on the human relevance of the proposed MOA: Data presented by the registrant are insufficient to conclude that the MOA for rodent hepatocellular carcinogenesis is not relevant to humans. CARC considers the proposed modes of action through the activation of multiple nuclear receptors to be relevant to humans.

CARC's Overall Conclusions on the Mouse Liver MOA: The CARC agrees that the weight of the evidence supports a mitogenic MOA for liver tumors in mice. The proposed mitogenic MOA involves the co-activation of CAR, PXR and PPARα nuclear receptors in the liver. The collective activation of these receptors results in a proliferative response in the liver leading to the formation of hepatocellular tumors. The mechanistic data adequately demonstrate dose and temporal concordance of the key events supporting that this mitogenic MOA is operative in mice exposed to high doses of valifenalate. Alternative MOAs for liver tumors in mice were evaluated and sufficiently ruled out as potential MOAs for liver tumors in valifenalate treated mice.

V. COMMITTEE'S ASSESSMENT OF THE WEIGHT OF THE EVIDENCE

Mouse

Lung Tumors: Adenomas were observed in female mice at the highest dose tested; however, the tumors were not considered treatment related as the concurrent controls for the study were also

outside the presented historical control range, there was no pair-wise statistical significance when compared to control animals, and there were no concurrent non-neoplastic lesions observed in the lung tissue.

Liver Tumors: There was a statistically significant trend ($p< 0.01$) and a pairwise significance ($p<0.05$) for both hepatocellular adenomas and carcinomas, and combined adenomas and carcinomas at the highest dose tested in male mice; and a significant trend ($p< 0.01$) and a pairwise significance ($p<0.05$) for hepatocellular adenomas and combined adenomas and carcinomas in female mice. These incidences fall outside of the laboratory's presented historical control range. **The CARC considered the hepatocellular adenomas, carcinomas and combined adenomas and carcinomas in males as well as the hepatocellular adenomas and combined adenomas and carcinomas in females to be treatment-related.**

Adequacy of Dosing: There was no excess mortality or moribundity observed at any dose level in either sex. The doses were considered to be adequate since the highest dose approached the limit dose (657/756 mg/kg/day [M/F]) and non-neoplastic histopathological findings were observed at this and lower dose levels including increased absolute and relative liver weights, generalized hepatocyte hypertrophy, liver masses, pale areas, accentuated lobular patterns and eosinophilic foci in both sexes.

Rat

There were no treatment-related tumors in male or female rats. The doses tested were considered adequate and not excessive to assess carcinogenicity.

Mode of Action for Liver Tumors in Mice

The weight of the evidence supports a mitogenic MOA for liver tumors in mice involving the co-activation of CAR, PXR and PPARα nuclear receptors. The collective activation of these receptors results in a proliferative response in the liver leading to the formation of hepatocellular adenomas/carcinomas. The mechanistic data adequately demonstrate dose and temporal concordance of the key events supporting that this mitogenic MOA is operative in mice exposed to high doses of valifenalate. CARC considers this MOA to be relevant to humans.

VI. CLASSIFICATION OF CARCINOGENIC POTENTIAL

Classification and Quantification of Carcinogenic Potential

The CARC considered the following in its weight-of-evidence deliberation on assessing the carcinogenic potential of valifenalate:

1. The lung tumors seen in female mice were not considered treatment-related;

2. The hepatocellular tumors seen in male and female mice were considered treatment-related based on:

a. Statistically significant trends ($p<0.01$) and statistically significant pair-wise ($p<0.05$) comparisons at 657 mg/kg/day in male dose group for liver adenomas, carcinomas and combined adenomas and carcinomas.
b. Statistically significant trends ($p < 0.01$) and statistically significant pair-wise ($p<0.05$) comparisons at 756 mg/kg/day in female dose group for liver adenomas, and combined adenomas and carcinomas.
c. The tumor incidences at the high dose in both sexes exceeded the historical control ranges for hepatocellular tumors in this strain of mice.

3. There is no mutagenicity concern based on the results from the *in vitro* and *in vivo* genetic toxicity studies;

4. The proposed MOA (co-activation of the PPARα, CAR and PXR receptors leading to hepatocellular proliferation and liver tumors in mice) was adequately supported by studies that clearly identified the sequence of key events, dose-response concordance and temporal relationship for these high dose tumors; and

5. Data presented by the registrant are insufficient to conclude that the mitogenic MOA for rodent hepatocellular carcinogenesis is not relevant to humans.

In accordance with the EPA's *Final Guidelines for Carcinogen Risk Assessment* (March 2005), the CARC classified valifenalate as "Not Likely to be carcinogenic to humans at dose levels that do not cause a proliferative response".

VII. QUANTIFICATION OF CARCINOGENIC POTENTIAL

The Agency has determined that quantification of risk using a non-linear approach (i.e., reference dose (RfD) will adequately account for all chronic toxicity, including carcinogenicity, that could result from exposure to valifenalate.

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